Preface

    Interleukins are a family of proteins that regulate the maturation, differentiation, or activation of cells involved in immunity and inflammation, and belong to a broader family termed cytokines. Collectively these proteins are the key orchestrators of host defense and the response to tissue injury. There are currently 23 different interleukins (numbered from IL-1 to IL-23), although the full extent of the interleukin family will only become clear upon analysis of the human genome sequence. Most important, interleukins are central to the pathogenesis of a wide range of diseases that involve an immune component, including such conditions as rheumatoid arthritis, multiple sclerosis, ulcerative colitis, psoriasis, and asthma. Interleukins have also been implicated in other conditions, including cancer, migraine, myocardial infarction, and depression.

    In essence, when cells are activated by interleukins, a program of gene expression is initiated in the target cell that alters the cell's phenotype, leading to enhanced immune reactivity, inflammation, and/or proliferation. Interleukins are therefore at the core of the cellular basis for many diseases. They are the subject of intense investigation by biomedical researchers and the targeting or use of interleukins in the clinic is proceeding apace. Approaches such as targeting IL-4 in asthma or IL-1 in joint disease are being pursued, and it is likely that in the next 5–10 years a number of new therapies based on either inhibiting or administering interleukins will be available. In addition, the assaying of interleukins has a role in the diagnosis and prognosis of disease, and polymorphisms in interleukin genes may well be found to predispose individuals to disease.

    The basis for these many advances in interleukin research lies in the use of a range of methodologies for their study. In *Interleukin Protocols* we have brought together a critical mass of chapters covering the major techniques currently available to researchers in this area. The book is divided into five sections. Parts I and II concern a range of methods for assaying interleukin protein and mRNA. The ELISA is the mainstay of assaying interleukin protein production and the chapters here cover the basic methodologies, where to purchase reagents and also recent developments in the use of ELISA. The use

of FACS as a method of assaying interleukins intracellularly has been an important advance and is also covered. The ability to measure interleukin mRNA is another important technique in interleukin research and several chapters describe quantitative methods that mainly rely on RT-PCR and RNAse protection. Part II gives examples of how to measure specific interleukins in order to illustrate the approaches that can be used for investigators interested in a particular interleukin.

Part III covers the assays of interleukins in specific pathologies, including breast cancer, depression, psoriasis, Grave's disease, migraine, and myocardial infarction. Part IV is related to Part III in that it also concerns pathologies, but has as its focus the assaying of interleukins in different biological fluids relevant to disease. These include peritoneal fluids, sputum from asthma patients, synovial fluid from arthritic joints, and cerebrospinal fluid from patients with meningitis. More important, this section covers the difficulties associated with the measuring of interleukins in such fluids. Finally, Part V concerns newer methods in the study of interleukin signal transduction, analysis of polymorphisms in interleukin genes, and the use of cDNA arrays, areas that will surely expand greatly in the next years as the feasibility of assaying the consequences of interleukin action in disease becomes more apparent.

Interleukin Protocols will therefore be of interest to a wide range of investigators, from molecular and cell biologists to immunologists to clinical investigators. The discovery of interleukins and the analysis of their role in disease represent major advances in molecular medicine. The methods described will help researchers continue to advance, ultimately leading to better diagnosis, prognosis, and treatment of many diseases where there remains an unmet medical need.

Luke A. J. O'Neill
Andrew Bowie

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ELISPOT Technique for Assaying Interleukins

Almuth Christine Hauer and Mona Bajaj-Elliott

1. Introduction

The enzyme-linked immunosorbent assay (ELISA) spot (ELISPOT) procedure is basically a modification of the plaque techniques, hence its initial synonym ELISA plaque assay. Plaque assays allow the enumeration of antibody-secreting cells by diluting them in an environment in which the antibody formed by each individual cell produces a readily observable effect. Based on this principle, Czerkinsky and coworkers *(1)* first described a modification of this technique which could be used for the detection and enumeration of antibody-producing cells in vitro. In this modified assay, a suspension of single antibody-forming cells was incubated on a precoated solid phase, i.e., on a dish of immobilized antigen, to which specific antibody secreted during the incubation period would bind. This locally captured antibody was visualized after removal of the cells by treatment with an enzyme-conjugated anti-immunoglobulin and development of a color reaction by incorporating the substrate in a gel that was poured over the ground of the dish. Limited diffusion of the colored reaction product in the gel provided a series of macroscopic spots that were readily enumerated.

Since then the ELISPOT assay has been employed not only to enumerate specific as well as total immunoglobulin-secreting cells but also to detect a variety of cells secreting antigenic substances *(2)*. Although Versteegen et al. *(3)* were the first to use this assay to detect human cells secreting interferon-γ (IFN-γ), an important modification of the ELISPOT assay employing nitrocellulose membranes and epitope-specific monoclonal antibodies has made it more reproducible and sensitive in detecting cytokine-secreting cells at a single cell level *(4)*. The advantage of this modification is the use of nitrocellulose plates as solid support instead of the former polystyrene surfaces. As they have a

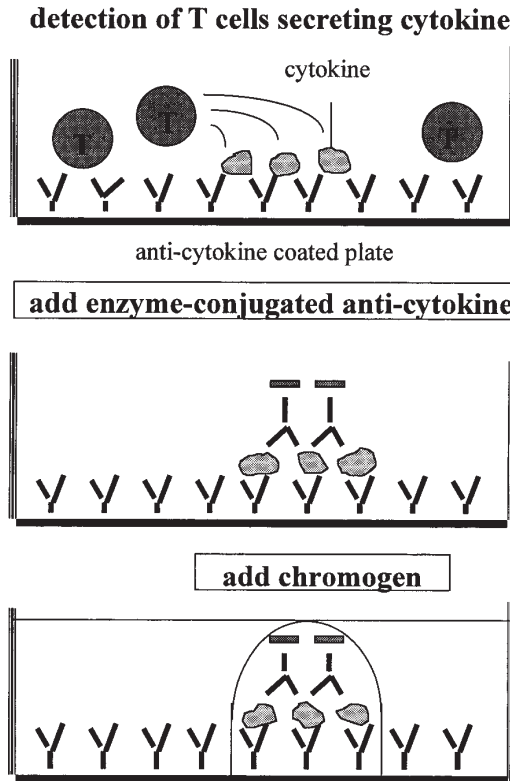## detection of T cells secreting cytokine

Fig. 1. Plates are coated with anti-cytokine and the captured cytokine is detected with enzyme-coupled antibody and visualized by addition of a chromogen.

higher protein binding capacity, requirements for relatively large amounts of expensive coating reagents can be minimized, and nitrocellulose plates are now routinely used for cytokine analysis by the ELISPOT method (*see* **Fig. 1**). Furthermore, this method became a well-established technique for measuring cytokine synthesis, as it also has the benefit allowing cells secreting different cytokines to be compared with a common denominator, namely, the cell number. However, there remains the disadvantage that one has very little idea of how much cytokine each cell is secreting. This is the reverse problem of assays measuring cytokines in supernatants: Here one knows the exact concentration of cytokine but not how many cells are secreting the molecule.

In principle, the ELISPOT method can be used in a whole variety of settings: Cytokine secretion by peripheral as well as mucosal lymphocytes can be measured, both the spontaneous secretion and that after in vitro stimulation

with various agents, but also after having positively or negatively selected for a number of cell subtypes, i.e., T-helper cells *(5,6)*. ELISPOT is equally used in settings involving animal experiments *(7)*. However, the technical details described in this chapter refer to work with human mononuclear cells only. The essential and overall determinant for the quality of the assay remains the initial preparation of the single cell suspension, i.e., the separation of peripheral lymphocytes or mucosal lymphocytes, and treatment of the cells before commencement of the assay.

2. Materials

2.1. Experimental Reagents and Buffers

This method requires sterile working conditions, and all steps should be performed in a laminar air flow hood. However, the final stage, including color development of the ELISPOTs, is performed at the bench.

The experimental reagents and buffers listed should be prepared as fresh as possible, but in convenient amounts; buffers such as phosphate-buffered saline (PBS), Tris-buffered saline (TBS), and the alkaline phosphate color substrate buffer can be prepared in advance (1-L aliquots) to the appropriate pH, filter-sterilized (0.2 μm), and stored until required. Reagent preparation, such as the dilution of antibodies and the preparation of the color substrate, should be done immediately before use. In case of the detecting secondary antibody, 1% fetal calf serum (FCS) has always to be added directly to the freshly prepared antibody.

If not otherwise stated, the general storage conditions are at 4°C for up to 2 mo.

1. RPMI-1640/10% FCS: RPMI-1640 serves as the principle cell medium for both peripheral and mucosal lymphocytes in this assay. It is also used for the individual cell dilutions. Supplementation with FCS is essential for cell viability: 10.3 g of RPMI-1640 powder (Sigma, Poole, Dorset, UK) and 2.0 g NaHCO$_3$ (20 mM; GIBCO, Paisley, Scotland) are dissolved in 900 mL MQ water and supplemented with 10% heat-inactivated FCS, 50 μL β$_2$-mercaptoethanol (Merck-BDH), 50 U/mL penicillin, 50 μg/mL streptomycin (ICN Flow), and 50 μg/mL Gentamicin (Roussel, Uxbridge, Middlesex, UK) and the pH adjusted to 7.4. The final volume is then made up to 1 L.
2. PBS: For 1 L of PBS, mix 7.02 g NaCl, 3.44 g Na$_2$HPO$_4$, and 0.79 g KH$_2$PO$_4$ and adjust to pH 7.4.
3. PBS/Tween-20: Tween-20 is a detergent, and its addition to the buffer reduces nonspecific binding of the antibodies, leading to low background. Tween-20 is stored at room temperature and should be added at a concentration of 0.05% (v/v) to the freshly prepared PBS.
4. TBS: 1 L TBS requires 8.0 g NaCl (Merck-BDH), 0.605g Tris(hydroxymethyl) methylamine (Merck-BDH), and 4.4 mL 1 N hydrochloric acid (Merck-BDH), adjusted to pH 7.6.
5. 5-Bromo-4-chloro-3-indoyl phosphate/nitro blue tetrazolium (BCIP/NBT) substrate solution (Bio-Rad, Hercules, CA): The reagents for the final step of the color development are obtained in a kit form consisting of:

a. Two substrates (substrate A and substrate B) that are stored at –20°C in the dark and are stable up to 12 mo. Both substrates should only be taken out of the deep freeze for as short a period as possible, ideally placed on ice.

b. An alkaline phosphatase substrate buffer that has to be prepared initially under sterile conditions following the manufacturer's instructions exactly, adjusted to pH 7.6 and filtered. It can be then stored at 4°C for up to 12 mo.

6. Ficoll-hypaque (Amersham-Pharmacia, UK): Ficoll is used in the isolation of peripheral mononuclear cells by density gradient centrifugation. It is in liquid form, ready to use, and can be stored at 4°C for up to 2–4 mo.

7. Hanks' balanced salt solution (HBSS-CMF, free of calcium and magnesium; Flow Laboratories, McLean, VA): HBSS helps to dissociate the epithelia from the underlying mesenchyme by complexing with Ca^{2+} ions. It can be stored at 4°C for up to 6 mo.

8. Collagenase (Type 1, Sigma): Collagenase is needed for the enzymatic digestion of mucosal specimens. It comes as a powder and is stored at –20°C.

9. Cycloheximide (Sigma): Cycloheximide is a protein synthesis inhibitor and thus affects the cell function directly. It can therefore be used in control experiments to ensure that one does not measure cytokines released from the cytoplasm of dead cells or membrane-bound cytokines being released into the medium.

10. Trypan blue (0.1%, Bioconcept, Switzerland): Small amounts of freshly prepared single cell suspensions are mixed with trypan blue in order to count the mononuclear cells and to determine the viability of cells. Trypan blue should always be freshly filtered immediately before use using a microfilter. It is stored at room temperature (RT), in the dark, for up to 6 mo.

2.2. Monoclonal Antibodies

We routinely use this assay to detect the secretion of cytokines such as IFN-γ, interleukin (IL)-4, IL-5, IL-10, and transforming growth factor-β (TGF-β) by peripheral blood and mucosal lymphocytes. The concentrations of the primary and secondary antibodies listed below have been optimized for our system (*5,6,8,9*). Although the reader may have to test and modify these conditions for his/her experiment, the range of concentrations and dilutions suggested may be a good starting point.

The set of monoclonal antibodies (MAbs) for each cytokine includes a primary ("capture") and a secondary ("detecting") antibody. The nitrocellulose plates are initially coated with the primary MAb; the cells secreting the particular cytokine will affinity bind to it, hence "capture." The secondary MAb is biotin labeled and recognizes a different epitope on the cytokine, thus leading to binding and sandwich formation with the antibody-cytokine complex. A further MAb, namely, streptavidin alkaline phosphatase, that binds to the biotinylated secondary MAb is always added, thus acting as the "universal" tertiary antibody of the assay.

All MAbs listed are stored in the dark at 4°C. They are diluted as indicated in their respective buffers immediately before use, resulting in a final

concentration of MAb per culture well that equals 0.5–0.7 μg/100 μL (0.5–0.7 μg/culture well) for all the primary antibodies and 0.15–0.3 μg/100 μL (0.15–0.3 μg/culture well) for all the secondary antibodies.

1. IFN-γ antibodies (Chromogenix AB, Mölndal, Sweden): Stock concentrations are 100 μg/mL (capture) and 30 μg/500 μL (detecting).
2. IL-4 antibodies (Mabtech, Stockholm, Sweden): Stock concentrations are 100 μg/100 μL (capture and detecting).
3. IL-5 antibodies (Pharmingen, San Diego, CA): Stock concentrations are 500 μg/mL (capture and detecting).
4. IL-10 antibodies (Pharmingen): Stock concentrations are 500 μg/mL (capture and detecting).
5. TGF-β antibodies (R & D Systems, Minneapolis, MN): Stock concentrations are 500 μg/mL (capture) and 50 μg/mL (detecting).
6. Streptavidin alkaline phophatase (Mabtech): 1 mL.

3. Methods

ELISPOT is essentially an assay that analyzes the cytokine-secreting capacity of viable cells only! One should therefore proceed with the experiment as quickly as possible to keep the yield of viable cells high, i.e., within a maximum of 2–4 h after having obtained either blood sample or mucosal specimen. Cells have to be carefully worked with, and, after single cell separations have been prepared, cells should be always kept cold, on ice.

3.1. Separation of Peripheral Blood Mononuclear Cells

Peripheral blood mononuclear cells (PBMCs) are obtained from venous heparinized blood layered over a Ficoll-Hypaque density gradient using standard procedures *(8)*:

1. A minimum of 2 mL of whole blood is mixed 1:1 with sterile RPMI-1640/10% FCS (i.e., 2 mL of RPMI-1640/10% FCS) and carefully layered over the same amount of Ficoll (i.e., 4 mL of Ficoll; *see* **Note 1**).
2. The blood layered over Ficoll is spun down in the centrifuge at 1500 rpm, *20°C*, *without brake* for 20–25 min (*see* **Note 2**).
3. Cells of the buffy coat (i.e., the interphase mononuclear cells) are carefully collected **(Fig. 2)** using a Pasteur pipet with a small rubber bulb adjusted (*see* **Note 3**), resuspended in 10–15 mL of RPMI-1640/10%FCS and washed three times at 1500 rpm, *4°C*, *with maximal brake*, for 10 min.
4. After resuspension in 1 mL of medium, the cell number is determined **(Fig. 3)** using a hemocytometer (*see* **Note 4**).
5. Viable cells are identified by trypan blue exclusion and should comprise more than 95% before use (*see* **Note 5**).
6. To specify the subtypes of the PBMCs further, small samples can be prepared at this stage either for immunostaining or for fluorescence-activated cell sorting (FACS) analysis *(5)*.
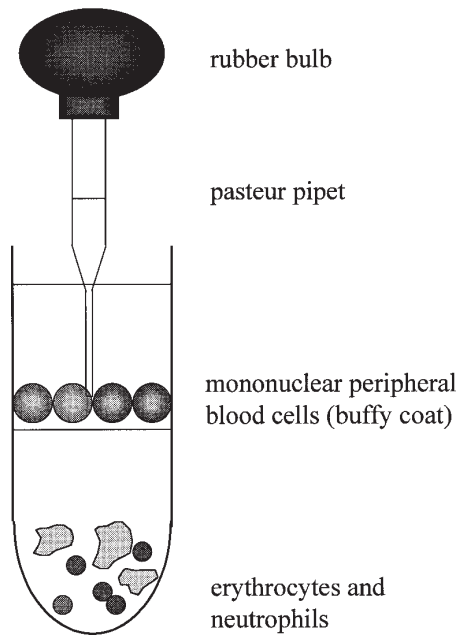
Fig. 2. Suctioning of the buffy coat by a Pasteur pipet.

3.2. Isolation of Mucosal Mononuclear Cells

Mononuclear cells are isolated from biopsies by an enzymatic digestion process.

1. Specimens should be placed in cold RPMI-1640/10% FCS (i.e., 2 pinch biopsies in 10–15 mL medium) directly after the biopsy has been performed and should be processed within 2–4 h.
2. Then specimens are put into 20 mL of HBSS for 45–75 min at RT and washed (10 min, 1500 rpm, 4°C, maximal brake). This process allows the separation of the epithelia.
3. The remaining samples are then placed into RPMI-1640/10%FCS using a Pasteur pipet, and 1.5–5 mg collagenase/mL medium is added (*see* **Note 6**).
4. Tubes are incubated at 37°C in a 5% CO_2/95% air/water-saturated atmosphere with vigorous pipetting every 10–15 min, using a pasteur pipet with a small rubber bulb attached.
5. The enzyme treatment is finished when tissue pieces are dissolved to an almost homogenous cell suspension. This takes usually 3–5 h.
6. Cells are washed twice in RPMI-1640/10% FCS and resuspended in 10 mL of medium. Facultatively, they can then be filtered through a glass wool column (*see* **Note 7**).
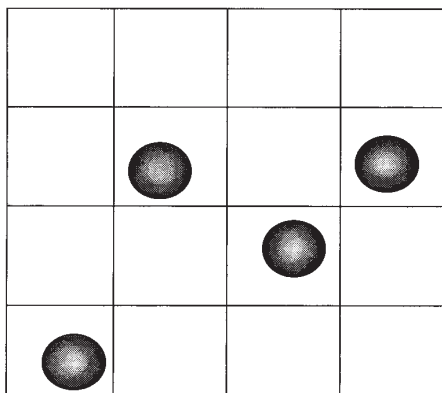
4 MNC*/16 hemocytometer squares =
0.4 x 10^6 MNC/ml
(10 µl cell suspension + 90 µl trypan blue)

* MNC: mononuclear cells

Fig. 3. Example for the determination of the cell number per volume using a hemocytometer.

7. After a final wash, cells are resuspended in 500 µL of RPMI-1640/10% FCS, counted, and left on ice while checking for viability (>95%) and purity (<5% epithelial cells) (*see* **Note 8**).
8. Cytocentrifuge preparations can be performed at this stage (a minimum of two per sample, with 5–10,000 cells/slide) and stored until immunostaining.

3.3. Enumeration of IFN-, IL-4-, IL-5-, and IL-10-Secreting Cells

Both diluted antibodies and single cell suspensions are always added in 100-µL vol to each well, using individual pipets. All washing steps are performed with 200-µL vol of the respective buffers, using a multichannel pipet. One should always perform assays involving peripheral blood mononuclear cells (MNCs) at least in triplicate or quadruplicate, adding 100,000–150,000 MNC/culture well, and assays with mucosal MNCs at least in duplicate, adding 5000–50,000 MNC/well.

1. Nitrocellulose-bottomed microtiter wells (Millipore Bedford, MA) are coated for 3 h at RT with the cytokine-specific monoclonal capture antibody, diluted in 100 µL sterile PBS per well (*see* **Note 9**). Plates should be agitated for 1–2 min directly after adding the antibody to ensure homogenous suspension.
2. After 3 h, unadsorbed antibody is removed by three washes with PBS (*see* **Note 10**).
3. Single cell suspensions in 100-µL vol are added to each well (*see* **Note 11**).

4. Cells are incubated for 20 h (or overnight) at 37°C in a humidified atmosphere of 5% CO_2 in air.
5. The cells are removed by washing the wells eight times with PBS/0.05% Tween.
6. A biotin-labeled cytokine-specific MAb is then used as a detecting antibody and added in 100-μL vol/well.
7. The plate is again agitated for 1–2 min and then left for an incubation period of 3 h at RT (*see* **Note 12**).
8. Wells are washed 6× with PBS/0.05% Tween.
9. Streptavidin alkaline phophatase is diluted 1:1000 in TBS and added at 100 μL/well; after agitation, the plate is incubated for 1 h at 37°C in the CO_2 incubator (*see* **Note 13**).
10. Wells are washed five times with TBS and finally once with the alkaline phosphatase substrate buffer.
11. Color substrate is freshly prepared (10 μL of substrate A and 10 μL of substrate B/1 mL of substrate buffer) and added in 100-μL aliquots to each culture well.
12. The plates are left at RT until dark blue spots appear (20–40 min).
13. The enzyme reaction is stopped by washing three times with distilled water. The wells are allowed to dry and the number of spots per well enumerated using a dissecting microscope (×25 magnification; *see* **Note 14**).
14. ELISPOT sizes can be determined by measuring the spot diameters on the basis of their optical density by computer-assisted image analysis (SeeScan, Cambridge, UK). With this equipment spots are visualized on a monitor and their diameter measured using a mouse-controlled cursor (*see* **Note 15**).

3.4. Control Experiments

To assess true spot formation, inhibition assays with cycloheximide can be performed. Cycloheximide inhibits protein synthesis, leading to subsequent downregulation of cytokine synthesis and secretion, and thus spot formation. This time-course experiment includes the following steps:

1. The single cell suspension of blood or mucosal lymphocytes, respectively, is assessed for its cell number, resuspended in 20–40 mL RPMI-1640/10% FCS, and aliquoted into four culture flasks (50-mL vol).
2. Two flasks are incubated for 3 h, and the other two for 5 h, with one at each time containing cycloheximide at a final concentration of 50 ng/mL.
3. After two subsequent washes, cell suspensions are added at 100-μL vol to the previously coated culture wells and the ELISPOT assay continued as usual.
4. Inhibition of spot formation by cycloheximide within a time-course of 3 and 5 h, respectively, can finally be calculated by comparing the spot numbers of wells containing cell suspensions with and without cycloheximide added.

3.5. Stimulation of Mononuclear Cells for the ELISPOT Assay

Both peripheral blood and mucosal MNCs can be stimulated before using them in the ELISPOT assay as usual. However, stimulation assays involving mucosal

MNCs will always depend on a sufficiently high cell yield at the beginning. Stimulants can either serve as positive controls (i.e., T-cell mitogens such as phytohemagglutinin) or in assays analyzing cytokine secretion upon stimulation with specific antigens (i.e., dietary antigens such as various cow's milk proteins).

1. A single cell suspension is prepared as usual, counted, tested for viability, and resuspended in 20 mL RPMI-1640/10% FCS.
2. This cell suspension is then aliquoted (i.e., 100,000 peripheral MNCs and 10–50,000 mucosal MNCs/aliquot). One aliquot serves as the unstimulated control and the remaining aliquots for adding the respective stimulating agents. All are incubated in culture flasks or 24-well plates in the CO_2 incubator overnight.
3. Cells are then washed, checked for viability, counted, and added to the previously coated wells in 100-µL vol, before the ELISPOT assay is continued as usual.

3.6. Using Selected Cell Subtypes

Single cell suspensions that have either been depleted of or enriched for a given cell population by magnetic separation techniques can equally be used for the ELISPOT assay.

1. A minimum of three wells per cytokine (ideally six wells for duplicate experiments) has to be coated in advance to ensure that the unselected cell fraction along with both the negative and the positive cell fraction can be set up.
2. After the separating procedures, cells have again to be counted, tested for viability, resuspended in cell medium, and added at 100-µL vol to the wells in order to continue with the assay as usual (**Figs. 4** and **5**).

4. Notes

1. This layering can be easily performed using a broad-end Pasteur pipet with a small rubber bulb in order to suck up the milliliter amounts of the suspended blood gently and repetitively and place them onto the Ficoll.
2. Because too much mixing of the layers will dramatically reduce the yield of lymphocytes, care is required when transferring the tube to the centrifuge. No brake on this centrifugation is recommended, as this helps to maintain the gradient established.
3. One should proceed with the Pasteur pipet directly to the buffy coat (the mononuclear cell layer) that appears at the interphase between the top (aqueous) and the bottom (Ficoll) layer and suck it up as complete as possible trying not to disturb the respective layers in the least (**Fig. 2**).
4. Cell suspension (10 µL) is diluted in 90 µL of trypan blue (0.1%) and quickly vortexed; a small amount of the mixture is then added to a hemocytometer. If there are enough cells, a minimum of 16 squares is counted; otherwise one should count 64 squares (example: 25 MNC/16 squares = 2.5×10^6/mL MNC; 136 MNC/16 squares: 13.6×10^6/mL MNC; **Fig. 3**).
5. This step has to be performed very fast, since trypan blue affects the viability of cells.
6. The amount of collagenase added can be adjusted individually according to one's experience through the experimental series. We found an amount of 2–2.5 mg/mL
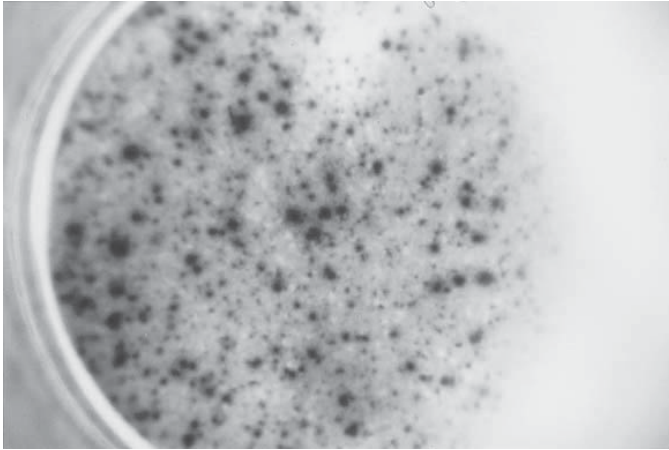
Fig. 4. Interleukin-4 ELISPOTS upon stimulation with β-lactoglobulin, by peripheral blood mononuclear cells (original magnification ×25).

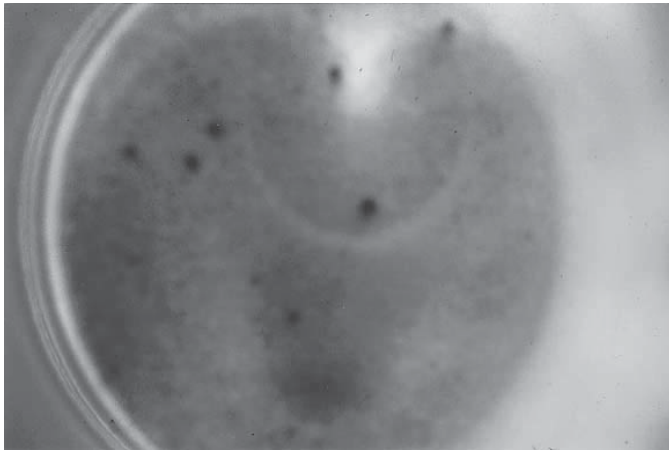Fig. 5. Transforming growth factor-β ELISPOTS by peripheral blood mononuclear cells (original magnification ×25).

collagenase appropriate when dealing with Peyer's patches, whereas specimens from the ileal mucosa needed up to 5 mg collagenase/mL to digest well. However, prolonged exposure to collagenase will affect the viability of cells. Once single cell suspension is obtained, proceed rapidly to the next step.

7. This filtering procedure might be useful in getting rid of remaining mucus and cell debris. However, loss of viable cells is also likely. Therefore a balance needs to be achieved between the requested cell number and the purity of the cell suspension.

8. Of 2 mL blood one can expect between 1.0 and 10.0×10^6 MNC/mL, depending on the patient's age (i.e., newborns have much higher MNC numbers than adults), and of one pinch biopsy between 0.2 and 1.5×10^6 MNC/mL. In case of small mucosal specimens, there are often only few remaining cells. To count these, it is therefore easier to resuspend them in 200 μL of medium only and to have thus a higher concentration of cells per volume. One has then to adjust the final cell count with respect to the diluting volume chosen (i.e., a count of 1.0×10^6 MNC diluted in 200 μL equals 0.2×10^6 MNC diluted in 1000 μL).

9. To match the antibody concentration required per well, the aforementioned antibodies have to be diluted as follows:

    a. Primary ("capture") antibodies:

        IFN-γ: 1:10   (80 μL MAb/800 μL PBS for 8 wells = 100 μL diluted MAb/well)

        IL-4, IL-5, IL-10: 1:50   (16 μL MAb/800 μL PBS for 8 wells = 100 μL diluted MAb/well)

        TGF-β: 1:167   (4.8 μL MAb/800 μL PBS for 8 wells = 100 μL diluted MAb/well)

    b. Secondary ("detecting") antibodies:

        IFN-γ: 1:10   (80 μL MAb/800 μL PBS/0.05% Tween, supplemented with 8 μL FCS for 8 wells = 100 μL diluted MAb/well)

        IL-4, IL-5, IL-10: 1:50

        TGF-β: 1:714

10. Antibody is removed by swiftly reversing the plates onto absorbing tissue covering some of the bench. From now on, all working steps should be performed as fast as possible in order not to allow the bottoms of the culture wells to dry, as dried nitrocellulose will reduce the binding of the subsequent reagents. Better leave buffers of the final washes in the wells while preparing the necessary antibodies!

11. Assays involving blood lymphocytes should be performed at least in triplicate for each cytokine (about $1–1.3 \times 10^5$ cells/100 μL). Assays with mucosal cells should be done in duplicate. In contrast to previous reports *(8)*, we found it worthwhile not only to reduce the input of mucosal cells per well, but also to modify the cell input depending on the respective cytokines tested. Since spontaneous secretion of IFN-γ proved to be very high in our studies *(5,6)*, one might add only $0.5–1.0$ instead of $5.0–7.0 \times 10^4$ MNCs/well. Due to this variation, the enumeration of spot-forming cells is more precise and easier to reproduce, since artifactual background staining is minimized. For measurement of IL-4-, IL-5-, and IL-10-secretion, the appropriate cell input ranges from 1.5 to 4.0×10^4/well.

12. It is very important not to put the plates into the CO_2 incubator by mistake, because this can destroy the experiment.

13. It is important that the duration of this incubation be not less than 45 min and not more than 75 min.

14. The frequency of spot-forming cells (SFC)/10^5 input MNCs is derived based on the number of cells placed into the wells. The mean of the duplicate or triplicate experiments, respectively, should be taken as a result. The reproducibility and accuracy of the counts should always be double-checked by a second observer, ideally blinded. If there are abundant spots, a precise count is impossible, but in order to have at least a semiquantitative result, one should only count a quarter of the well's ground and multiply it by four. In such cases, it is worthwhile to blind oneself and do repetitive counts. One should absolutely have an interobserver comparison in such situations.

15. This determination depends on whether such equipment is at hand. One can then follow the technical instructions and the menu for "measurement of optical density." In wells with many homogenously distributed spots, a minimum of 20 random spots should be measured, whereas in wells with fewer spots, each single spot should be analyzed.

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